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ILO 4/00181

Glycosylation and Processing of High Levels of Active Human Glucocerebrosidase in Invertebrate Cells Using a Baculovirus Expression Vector

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ABSTRACT

A human cDNA containing the complete coding region for the lysosomal glycoprotein glucocerebrosidase (EC 3.2.1.45) was introduced into the genome of *Autographa californica* nuclear polyhedrosis virus downstream from the polyhedrin promoter. Infection of *Spodoptera frugiperda* cells (SF9) with recombinant virus produced high levels of glucocerebrosidase, 40% of which was in the culture medium. The amino-terminal amino acid sequence of the recombinantly produced enzyme was identical to that of mature, human placental glucocerebrosidase, demonstrating that the signal sequence of the human preenzyme was recognized and appropriately removed in the SF9 invertebrate cells. The glucocerebrosidase in both the culture supernatant and SF9 cell pellet was glycosylated and contained, in part, high mannose oligosaccharide. These results demonstrate that insect cells can be used to produce abundant quantities of active mature human glucocerebrosidase that contains high mannose oligosaccharide as a consequence of post-translational processing.

INTRODUCTION

MUTATIONS IN THE GENE for the lysosomal enzyme glucocerebrosidase (EC 3.2.1.45, β-D-glucosyl-N-acylsphingosine glucohydrolase) result in the sphingolipidosis called Gaucher disease (Ginns *et al.*, 1984; Tsuji *et al.*, 1987). Although the biochemical defect is present in all tissues, the accumulation of the neutral glycosphingolipid glucosylceramide occurs predominantly within reticuloendothelial cells. On the basis of clinical signs and symptoms, Gaucher disease has been divided into three major phenotypes: type 1, non-neuronopathic; type 2, acute neuronopathic; and type 3, chronic neuronopathic (Barranger and Ginns, in press). Despite the stereotypic presentation of type 2 disease, within the type 1 and type 3 phenotypes there is marked variation in age of symptom onset and severity as well as in organ involvement. To understand more fully the biochemical basis for the heterogeneity seen in the dif-

ferent phenotypes of Gaucher disease, investigators have focused their attention on both biochemical and structural characterizations of normal and mutant glucocerebrosidases (Ginns *et al.*, 1982, 1983; Aerts *et al.*, 1985; Fabbro *et al.*, 1987; Graves *et al.*, 1986).

Although the amino acid sequence of normal human glucocerebrosidase has been determined by both direct chemical sequencing of placental glucocerebrosidase (B. Martin, unpublished data) and from cDNA (Ginns *et al.*, 1984, 1985; Sorge *et al.*, 1985; Tsuji *et al.*, 1986) and genomic DNA (Choudary *et al.*, 1985) deduced amino acid sequences, neither the active site nor other functional domains of even the normal enzyme have been well characterized (Grabowski *et al.*, 1984, 1985b). However, it has been demonstrated that the mutations causing Gaucher disease result in catalytically defective glucocerebrosidases (Brady *et al.*, 1965; Patrick, 1965) that differ from the normal enzyme in stability (Jonsson *et al.*, 1985), post-transla-

tional processing (Ginns et al., 1982, 1983, 1984; Erickson et al., 1985; Grabowski et al., 1985a; Jonsson et al., 1987), and/or compartmentalization (Willemsen et al., 1987). Although some of these allelic mutations have been documented by sequencing type 1 (Tsuji et al., in press) and type 2 (Tsuji et al., 1987) genomic DNAs for glucocerebrosidase, sufficient quantities of homogeneous mutant enzyme have not been available to carry out extensive structural analyses.

To determine whether sufficient glucocerebrosidase could be produced recombinantly for both structural analyses and in vivo therapeutic evaluations, we investigated the feasibility of producing abundant quantities of normal human glucocerebrosidase using a recombinant baculovirus derived from Autographa californica nuclear polyhedrosis virus (AcNPV) in eukaryotic insect cells (Smith et al., 1985; Matsuura et al., 1987; Maeda et al., 1985).

Under transcriptional control of the polyhedrin promoter in the recombinant nuclear polyhedrosis virus, the normal human glucocerebrosidase cDNA directed the synthesis of abundant quantities of glycosylated, active enzyme. In this report we compare structural and biochemical properties of the native human placental enzyme with those of the SF9 cell pellet and culture supernatant forms of glucocerebrosidase produced using the baculovirus-derived expression vector.

MATERIALS AND METHODS

Materials

Restriction endonucleases and recombinant enzymes were obtained either from Life Science Technologies or New England Biolabs. Concanavalin A-Sepharose was obtained from Pharmacia. Octyl- and decyl-agarose were purchased from ICN Biomedicals, Inc. Polyvinylidene difluoride (PVDF) membranes, 0.45 μm pore size, were obtained from Millipore Corp. Sequencer chemicals and solvents for on-line PTH analysis were purchased from Applied Biosystems Inc. Endoglycosidase-H was from Miles Scientific while N-glycanase was purchased from Genzyme Corp.

Construction of recombinant baculoviruses

Spodoptera frugiperda SF9 cells, plasmid pAc373, and wild-type AcNPV strain E2 were obtained from Max Summers (Texas A & M University). The SF9 cells were maintained in culture at 28°C using TNM-FH media (GIBCO) (Hink, 1970). The cDNA for human glucocerebrosidase was obtained from plasmid pUC19/GC, a derivative of an Okayama-Berg clone from a SV40-transformed human fibroblast cDNA library (Okayama and Berg, 1983). This cDNA contained 5' and 3' untranslated sequences as well as the complete coding region for glucocerebrosidase. As shown in Fig. 1, pAc373/GC was generated by ligation of the blunted Eco RI-Xba I fragment from pUC19/GC into the blunted unique Bam HI site of pAC373. Correct orien-
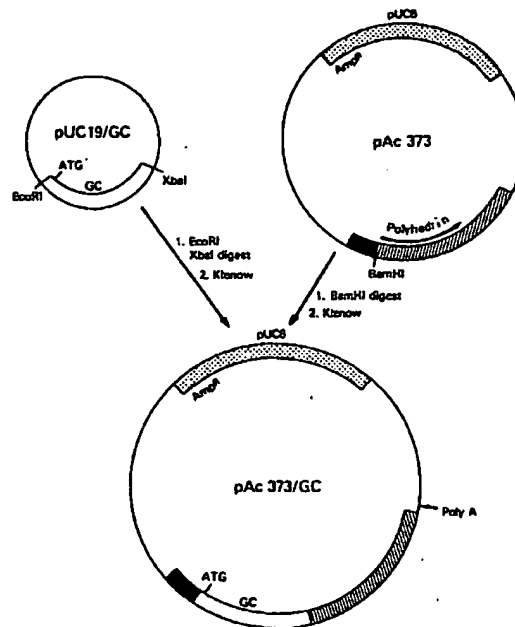

FIG. 1. Construction of the baculovirus-derived vector pAc373/GC containing human glucocerebrosidase cDNA. The Eco RI-Xba I fragment from pUC19/GC that contained the complete coding region for human glucocerebrosidase was blunted with Klenow polymerase. This insert, containing both the mature enzyme and signal peptide coding sequences, was ligated in a 5' to 3' orientation into the Bam HI site (also blunted with Klenow) of pAc373 downstream from the polyhedrin promoter.

tation of the glucocerebrosidase cDNA insert was determined by restriction endonuclease analysis.

Recombinant baculovirus containing the human glucocerebrosidase coding sequence under transcriptional control of the polyhedrin promoter was produced by cotransfection of wild-type virus, AcNPV, with plasmid pAc373/GC into SF9 cells (Summers and Smith, 1987). Five to six days after cotransfection, virus was harvested from the culture supernatant and used to inoculate new monolayers of SF9 cells in Petri dishes that were subsequently overlaid with 1.5% low-melting agarose containing TNM-FH medium. Seventy-two hours later, the agarose overlay was removed and stored at 4°C, and the cell monolayer was blotted onto a nitrocellulose disk (BA85, Schleicher & Schuell). The disk was hybridized to the random primed ^{32}P-labeled Eco RI-Xba I glucocerebrosidase cDNA fragment from pUC19/GC (Wahl et al., 1979; Feinberg and Vogelstein, 1983). Areas on the agarose overlay corresponding to points on the nitrocellulose disk showing hybridization signal were excised and placed in 1 ml of TNM-FH medium. This virus was used for infection of SF9

monolayer cultures, and an additional five cycles of infec-
tion-hybridization were carried out during the plaque puri-
fication.

Enzyme purification

Recombinantly produced glucocerebrosidase was par-
tially purified using a modification of the procedure de-
scribed by Furbish et al. (1977). Cell culture supernatants
were precipitated with 195 g/liter ammonium sulfate. SF9
cell pellets containing the recombinantly produced gluco-
cerebrosidase were extracted into 25 mM sodium phos-
phate buffer pH 6.5, containing 150 mM NaCl and 0.1%
Triton X-100, followed by sonication twice at 50 W for 10
sec. After precipitation with ammonium sulfate (195 g/
liter), the resuspended pellets were extracted with n-buta-
nol as described, but ultrafiltration using a YM30 mem-
brane (Amicon) replaced dialysis. After decyl- and octyl-
agarose hydrophobic interaction chromatography at room
temperature, the fractions containing glucocerebrosidase
activity were pooled, and the ethylene glycol concentration
was reduced using an Amicon ultrafiltration cell fitted with
a YM30 membrane.

Carbohydrate characterization

Endoglycosidase-H was dissolved in 100 mM sodium
acetate pH 6.0 at a final concentration of 10 U/ml. N-Glyc-
anase was supplied as a 250 U/ml suspension in 50% glyc-
erol. Either human placental enzyme or 50-μl aliquots of
the decyl-agarose fractions containing glucocerebrosidase
activity were adjusted to 0.5% NaDodSO$_4$/1 M β-mercap-
toethanol and boiled for 2 min. The samples were then di-
luted with appropriate buffer to either 200 mM sodium
acetate pH 6.0 (for endoglycosidase-H) or 200 mM sodium
phosphate pH 8.5 (for N-glycanase) and a final composition
of 0.1% NaDodSO$_4$, 0.7% NP-40, and 0.02 M β-mercap-
toethanol (Trimble and Maley, 1984; Tarentino et al.,
1985). The samples were again boiled for 1 min, and then
endoglycosidase-H and N-glycanase were added to final
concentrations of 50 mU/ml and 20 U/ml, respectively.
Digestions were for 16 h at 37°C. Carboxypeptidase Y was
used as a control for both deglycosylation reactions.

Western blot analysis

NaDodSO$_4$-polyacrylamide gel electrophoresis and
Western blot analysis were performed as previously de-
scribed by Ginns et al. (1982).

Amino acid sequence analysis

Samples used for amino acid sequence analysis were
electrophoretically fractionated on NaDodSO$_4$ polyacryl-
amide gels as described above and then transferred to
PVDF membranes as described by Matsudaira (1987).
Briefly, after electrophoresis, the gel was incubated in
transfer buffer (0.01 M CAPS, 10% methanol, pH 11.0)
for 10 min prior to transblotting (50 mA for 4 hr). The gel
was then washed with HPLC-grade water for 5 min,
stained with 0.1% Coomassie Blue R250 (in 50% metha-
nol) for 5 min, and finally destained for 10 min with 50%
methanol–10% acetic acid. The PVDF membrane was
again washed with HPLC-grade water, dried under a
stream of nitrogen, and stored in a sealed bag at −20°C
until used for amino acid sequencing.

Amino acid sequence analysis was accomplished using
an Applied Biosystems Model 470A gas-phase sequencer
equipped with a Model 120A on-line PTH-amino acid
analyzer. The program 03R PTH was used directly for se-
quencing without pretreatment of the membrane strip with
Polybrene. An approximately 2 × 8 mm piece of PVDF
membrane containing the protein band of interest (Fig. 4,
below) was excised, centered on the Teflon seal, and
placed in the cartridge block of the sequencer. Multiple
strips of the PVDF membrane could be stacked in this
manner, thus increasing the amount of protein available
for sequencing. The initial and repetitive yields for se-
quencing recombinant glucocerebrosidase were calculated
by comparison with the yields obtained after 100 pmoles of
human placental glucocerebrosidase were electrophoresed,
transblotted to PVDF, and subjected to 10 cycles of amino
acid sequence (Table 1).

Glucocerebrosidase assays

For pH profile and inhibition experiments, glucocere-
brosidase activity was measured using 100 mM potassium
phosphate buffer containing 0.15% Triton X-100, 2.5 μl of
β-D-[1-^{14}C]glucocerebroside (7.5 mg/ml in sodium tauro-
cholate at 50 mg/ml), and sample in a total volume of 200
μl (Ginns et al., 1982). Preincubations with conduritol-B-
epoxide were for 30 min at 37°C (Legler, 1977). For K_m
determination, β-glucosidase activity was assayed at pH
5.9 using the artificial substrate 4-methylumbelliferyl-β-D-
glucopyranoside (4MUGP) in 100 mM potassium phos-
phate buffer containing 0.15% Triton X-100 and 0.125%
sodium taurocholate (Peters et al., 1976; Wenger et al.,
1978). Purification of recombinant glucocerebrosidase was
also monitored using 4MUGP.

RESULTS

Expression of human glucocerebrosidase in SF9 cells

Cotransfection of SF9 cells with pAc373/GC (Fig. 1)
and wild-type AcNPV DNA using a modification of the
calcium phosphate precipitation technique (Graham and
van der Eb, 1973) generated recombinant baculovirus that
contained human glucocerebrosidase cDNA. As early as 5
days after the initial infection, Western blot analysis of
culture medium demonstrated the presence of cross-reac-

TABLE 1. REPETITIVE YIELD OF RECOMBINANT AND PLACENTAL HUMAN
GLUCOCEREBROSIDASE DURING SEQUENCE ANALYSIS

	A	R	P	–	I	P	K	S	F	G	Repetitive yield[a] (%)
				Initial yield 77%							
Amt.[b] (pmole): placental enzyme	77	14	49	–	43	31	23	11	40	45	92
Amt.[b] (pmole): enzyme in media	14	7	14	–	9	10	7	2	10	10	90
Amt.[b] (pmole): enzyme in cell pellet	10	2	6	–	3	4	1	tr	3	2	90

[a] Repetitive yield was calculated from P3 to P6..
[b] Amount transblotted was estimated by comparison to the intensity of bands obtained by transfer of 100 pmoles of human placental glucocerebrosidase (media 18 pmoles; cell pellet 13 pmoles).

tive material (CRM) to human glucocerebrosidase (data not shown) that was similar to the pattern shown in Fig. 2, lane M4. Recombinant baculovirus was subsequently plaque-purified as described above and used to infect SF9 invertebrate cells. Seventy-two hours postinfection, the SF9 cell pellets and culture medium were harvested. Western blot analysis of both recombinantly produced enzyme following decyl-agarose hydrophobic chromatography and purified human placental glucocerebrosidase is shown in Fig. 2, lanes P1, M4, and C7. Prominent bands of cross-reactive material to human glucocerebrosidase were seen at 59 kD in the culture medium (Fig. 2, M4) and at 65 kD in the cell pellet extract (Fig. 2, C7) lanes. Both the cell pellet and culture medium-associated glucocerebrosidase CRM showed a minor band at 52 kD. In contrast, human placental glucocerebrosidase CRM showed a major band at 65 kD. These apparent molecular weights for recombinant human glucocerebrosidase are in good agreement with those previously reported from Western blot (Ginns et al., 1982) and pulse-chase (Erickson et al., 1985; Jonsson et al., 1987) analyses of human tissues and cell lines. Western blot analysis of SF9 cells infected only with the wild-type baculovirus did not show any CRM to human glucocerebrosidase.

Recombinant glucocerebrosidase is active in SF9 cells.

The eukaryotic SF9 cells infected with recombinant baculovirus and the culture medium were harvested 72 h postinfection. From 1.2 × 10^{8} SF9 cells infected with recombinant baculovirus, the cell extract and culture medium (200 ml) contained 400,000 units and 270,000 units of glucocerebrosidase activity, respectively. After the decyl-agarose hydrophobic chromatography step in the purifica-

tion procedure, from 15 to 40% of this initial enzyme activity was recovered. The invertebrate eukaryotic SF9 cells infected with only wild-type baculovirus showed no significant glucocerebrosidase activity, consistent with the absence of enzyme CRM.

The pH profiles of human placental and recombinantly produced human glucocerebrosidases are shown in Fig. 3. In all three cases, peaks of activity are observed between pH 4.5 and 5.0 and pH 5.5 and 6.0. The K_m values for the recombinant glucocerebrosidase from the cell pellet and culture supernatant were 3.6 mM and 3.3 mM, respectively. After 30 min of incubation at 37°C with the specific catalytic site inhibitor conduritol-B-epoxide, the placental as well as recombinant cell pellet and culture supernatant glucocerebrosidase activities were greater than 95% inhibited at both pH 4.5 and pH 5.5.

Glycosylation of glucocerebrosidase in SF9 cells

Since the post-translational processing of human glucocerebrosidase normally involves carbohydrate modification (Takasaki et al., 1984; Erickson et al., 1985; Furbish et al., 1981; Aerts et al., 1986; Murray et al., 1985), we investigated the pattern of glycosylation in SF9 cells. All recombinantly produced glucocerebrosidase activity in both the SF9 cell pellet and culture medium bound to Concanavalin A-Sepharose at 4°C, and could be eluted with α-methyl-mannoside at 37°C (data not shown).

Treatment of either human placental enzyme or the recombinant glucocerebrosidase in the culture medium or cell pellet (Fig. 2, lanes P2, M5, and C8, respectively) with endoglycosidase-H resulted in minor changes in the CRM pattern. However, incubation of the placental or recombinant glucocerebrosidases (Fig. 2, lanes P3, M6, and C9, re-
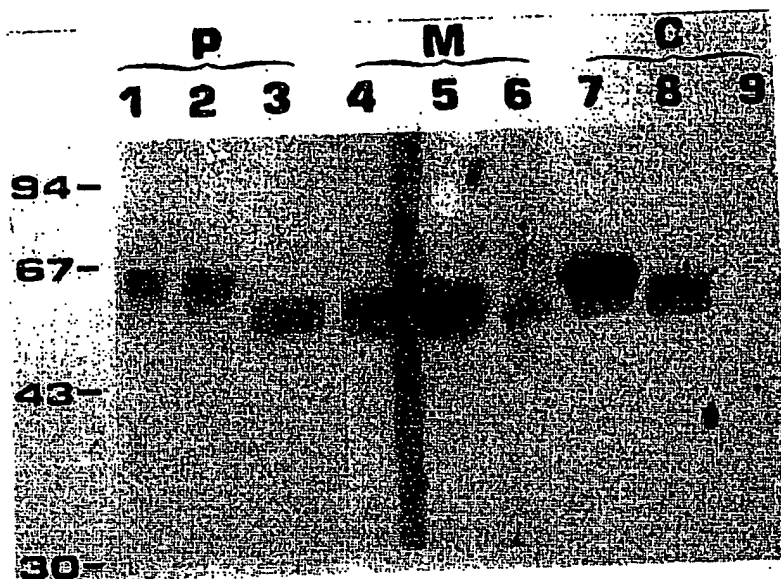
FIG. 2. Immunoblot detection of the recombinant baculovirus-encoded human glucocerebrosidase in infected SF9 cells. Sample preparation, electrophoresis, and Western blot analysis were performed as described in the text. Molecular weight size markers (M_r X10^{-3}) were phosphorylase b, albumin, ovalbumin, and carbonic anhydrase. P, Placental enzyme; M, media; and C, cell-associated. Western blots from untreated placental enzyme or recombinantly produced protein are shown in lanes P1, M4, and C7. Cross-reactive material (CRM) from endoglycosidase-H- and N-glycanase-digested samples are shown in lanes P2, M5, C8, and P3, M6, C9, respectively.

spectively) with N-glycanase produced a dramatic decrease in the apparent molecular weights of all three species, with the major CRM form appearing at approximately 52 kD in each case.

Sequence analysis of recombinant glucocerebrosidase

To determine whether the amino-terminal processing of recombinant glucocerebrosidase in the invertebrate eukaryotic SF9 cells is identical to that in human tissue, we sequenced the recombinant enzyme. Fractions from the decyl-agarose hydrophobic column that contained glucocerebrosidase activity were pooled, electrophoretically fractionated on polyacrylamide gels, and transblotted to PVDF membranes as described above. As seen from the Coomassie-stained PVDF membrane (Fig. 4, lanes 1 and 2) only two major protein bands were present. Western blot analysis confirmed that only the lower band contained CRM to human glucocerebrosidase (data not shown). Typically, approximately 2 μg of total protein per gel lane transferred to the PVDF membrane. The results obtained from sequencing placental and recombinant glucocerebrosidases are shown in Fig. 5. Each sample gave a single unique amino acid sequence. The amino-terminal sequence of recombinant glucocerebrosidases from the SF9 cell pellet and culture medium was identical to that of the purified human placental enzyme. The blank in position 4 of the re-

combinant enzyme sequences is consistent with cysteine because a cysteine at this position in the purified placental enzyme could only be identified after reduction and alkylation. The repetitive yields are shown in Table 1. From these data it is clear that the signal peptide of the preenzyme was correctly removed from the recombinant glucocerebrosidase produced in the invertebrate insect cells.

DISCUSSION

Our results demonstrate that high levels of active human glucocerebrosidase can be synthesized by the invertebrate SF9 cell-baculovirus expression system. It has been shown previously that the maturation of human glucocerebrosidase involves removal of a signal peptide (Ginns et al., 1985) followed by carbohydrate processing that involves the conversion of high mannose to complex oligosaccharide chains (Erickson et al., 1985). Microsequence analysis of recombinant glucocerebrosidase from both the SF9 insect cell pellet and culture supernatant demonstrated that the amino-terminal sequence was identical to that of human placental glucocerebrosidase (Tsuji et al., 1986). Importantly, the glycoprotein-processing mechanisms within the insect cells were able to recognize and appropriately cleave the mammalian signal peptide of glucocerebrosidase and produce a mature glycosylated protein having an
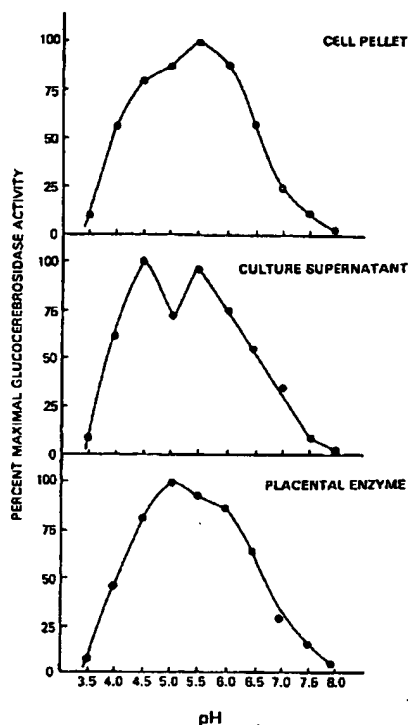
FIG. 3. Comparison of pH profiles of human placental glucocerebrosidase to recombinantly produced enzyme. Assays were performed using [^{14}C]glucocerebroside as described in the text.
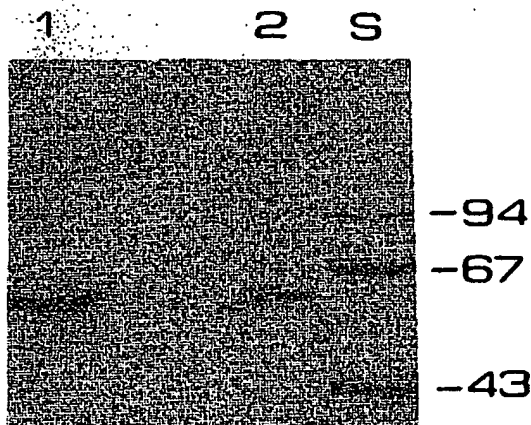
FIG. 4. Coomassie stain of polyvinylidine difluoride membrane (PVDF) containing electroblotted protein. Three micrograms of phosphorylase b, 4 μg of albumin, and 5 μg of ovalbumin were used as standards (lane S). Approximately 1 μg of partially purified recombinant glucocerebrosidase from infected SF9 cell pellets (lanes 1 and 2) was fractionated electrophoretically, transferred to the PVDF membrane, and stained with Coomassie, and amino acid sequence was determined as described.
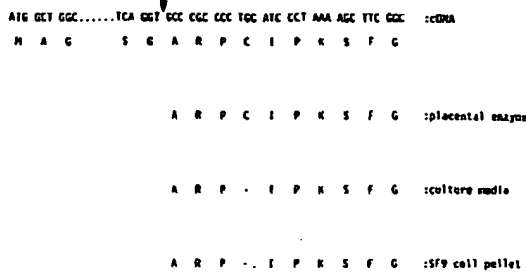
FIG. 5. Amino-terminal sequence of recombinant glucocerebrosidase in the SF9 cell pellet and culture supernatant. The mammalian peptidase cleavage site is indicated by the arrow. In the recombinant sequences, the blank (-) at position 4 is consistent with the presence of cysteine, since this amino acid was seen in the placental enzyme sequence only after reduction and alkylation of the sample.

amino-terminal sequence identical to that of human placental glucocerebrosidase.

Although the major bands of CRM to human glucocerebrosidase in the culture medium and cell pellet had apparent molecular weights of 59 kD and 65 kD (Fig. 2), respectively, both contained a minor band of CRM at 52 kD. The molecular weight of this minor band is consistent with the previously reported size of the unglycosylated form of human glucocerebrosidase, suggesting that a small proportion of the recombinantly produced enzyme does not contain carbohydrate (Erickson et al., 1985).

Previously, it had been demonstrated that glycosylation of human glucocerebrosidase and other glycoproteins in heterologous cells is species specific (Sheares et al., 1986; Choudary et al., 1986c). Therefore, it was reasonable to expect that the pattern of glucocerebrosidase CRM in SF9 invertebrate cells might not be identical to that seen in human tissues. Treatment of recombinantly produced enzyme with N-glycanase produced a single form at 52 kD (Fig. 2, lanes M6 and C9), confirming the presence of N-linked carbohydrate. The changes in the molecular weight of glucocerebrosidase CRM after endoglycosidase-H digestion (Fig. 2, lanes M5 and C8) indicate that human glucocerebrosidase produced in these invertebrate cells has at least some high-mannose oligosaccharide chains. These results also suggest that after the removal of the signal peptide, the post-translational processing of the recombinantly produced glucocerebrosidase in SF9 insect cells is similar to that in human tissues, and only involves carbohydrate modification (Erickson et al., 1985).

The pH profile of human glucocerebrosidase synthesized in insect cells using the baculovirus expression vector is very similar to that reported for glucocerebrosidase isolated from human tissues (Fig. 3) (Aerts et al., 1985). The glucocerebrosidase in the cell pellet and culture supernatant had K_m values of 3.6 mM and 3.3 mM, respectively,

close to that reported for normal human spleen glucocerebrosidase (K_m = 8 mM) (Basu et al., 1984). The recombinantly produced glucocerebrosidase was also appropriately inhibited by conduritol-B-epoxide, a specific active site inhibitor (Legler, 1977; Grabowski et al., 1985b). Based on a specific activity of 1.6 × 10⁴ U/mg for purified human placental glucocerebrosidase, approximately 2.2 mg of active recombinant enzyme could be produced in 2 days from 1 liter of insect cell-recombinant baculovirus culture (6.0 × 10⁸ cells).

Although a number of expression systems might be tried for production of large quantities of proteins (Choudary et al., 1986a, 1986b; Reiner et al., 1987), the structure and biological activity of the recombinant gene product should be as close as possible if not identical to the natural protein. Changes in post-translational modifications due to either the host cell processing machinery or to in vitro mutagenized cDNA changes could result in either increased or decreased biological function. This report demonstrates that the invertebrate baculovirus-SF9 cell expression system appropriately processes the mammalian signal sequence of human glucocerebrosidase, produces enzyme that is N-glycosylated, and can be used to rapidly produce large quantities of active human glucocerebrosidase.

ACKNOWLEDGMENTS

REFERENCES

AERTS, J.M.F.G., DONKER-KOOPMAN, W.E., VAN DER VLIET, M.K., JONSSON, L.M.V., GINNS, E.I., MURRAY, G.J., BARRANGER, J.A., TAGER, J.M., and SCHRAM, A.W. (1985). The occurrence of two immunologically distinguishable β-glucocerebrosidases in human spleen. Eur. J. Biochem. 150, 165–174.

AERTS, J.M., BRUL, S., DONKER-KOOPMAN, W.E., VAN WEELY, S., MURRAY, G.J., BARRANGER, J.A., TAGER, J.M., and SCHRAM, A.W. (1986). Efficient routing of glucocerebrosidase to lysosomes requires complex oligosaccharide chain formation. Biochem. Biophys. Res. Commun. 141, 452–458.

BARRANGER, J.A., and GINNS, E.I. (in press). Glucosylceramide lipidoses: Gaucher disease. In The Metabolic Basis of Inherited Disease, C.R. Scriver, A.L. Beaudet, W.S. Sly, and D. Valle, Eds., 6th ed. (McGraw-Hill, New York).

BASU, A., GLEW, R.H., DANIELS, L.B., and CLARK, L.S. (1984). Activators of spleen glucocerebrosidase from controls and patients with various forms of Gaucher's disease. J. Biol. Chem. 259, 1714–1719.

BRADY, R.O., KANFER, J.N., and SHAPIRO, D. (1965). Metabolism of glucocerebrosidase. II. Evidence of an enzymatic deficiency in Gaucher's disease. Biochem. Biophys. Res. Commun. 18, 221–225.

CHOUDARY, P.V., GINNS, E.I., and BARRANGER, J.A. (1985). Molecular cloning and analysis of the human β-glucocerebrosidase gene. DNA 4, 74.

CHOUDARY, P.V., HOROWITZ, M., BARRANGER, J.A., and GINNS, E.I. (1986a). Gene transfer and expression of active human glucocerebrosidase in mammalian cell cultures. DNA 5, 78.

CHOUDARY, P.V., BARRANGER, J.A., TSUJI, S., MAYOR, J., LAMARCA, M.E., CEPKO, C.L., MULLIGAN, R.C., and GINNS, E.I. (1986b). Retrovirus mediated transfer of the human glucocerebrosidase gene to Gaucher fibroblasts. Mol. Biol. Med. 3, 293–299.

CHOUDARY, P.V., TSUJI, S., MARTIN, B.M., GUILD, B.C., MULLIGAN, R.C., MURRAY, G.J., BARRANGER, J.A., and GINNS, E.I. (1986c). The molecular biology of Gaucher disease and the potential for gene therapy. Cold Spring Harbor Symp. Quant. Biol. 51, 1047–1052.

ERICKSON, A.H., GINNS, E.I., and BARRANGER, J.A. (1985). Biosynthesis of the lysosomal enzyme glucocerebrosidase. J. Biol. Chem. 260, 14319–14324.

FABBRO, D., DESNICK, R.J., and GRABOWSKI, G.A. (1987). Gaucher disease: Genetic heterogeneity within and among the subtypes detected by immunoblotting. Am. J. Hum. Genet. 40, 15–31.

FEINBERG, A.P., and VOGELSTEIN, B. (1983). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132, 6–13.

FURBISH, F.S., BLAIR, H.E., SHILOACH, J., PENTCHEV, P.G., and BRADY, R.O. (1977). Enzyme replacement therapy in Gaucher's disease: Large-scale purification of glucocerebrosidase suitable for human administration. Proc. Natl. Acad. Sci. USA 74, 3560–3563.

FURBISH, F.S., STEER, C.J., KREIT, N.L., and BARRANGER, J.A. (1981). Uptake and distribution of placental glucocerebrosidase in rat hepatic cells and effects of sequential deglycosylation. Biochim. Biophys. Acta 673, 425–434.

GINNS, E.I., BRADY, R.O., PIRRUCCELLO, S., MOORE, C., SORRELL, S., FURBISH, F.S., MURRAY, G.J., TAGER, J.M., and BARRANGER, J.A. (1982). Discrimination of neurologic and non-neurologic phenotypes of Gaucher disease. Proc. Natl. Acad. Sci. USA 79, 5607–5610.

GINNS, E.I., TEGELAERS, F.P.W., BARNEVELD, R., GALJAARD, H., REUSER, A.J.J., TAGER, J.M., BRADY, R.O., and BARRANGER, J.A. (1983). Determination of Gaucher's disease phenotypes with monoclonal antibody. Clin. Chim. Acta 131, 283–288.

GINNS, E.I., CHOUDARY, P.V., MARTIN, B.M., WINFIELD, S., STUBBLEFIELD, B., MAYOR, J., MERKLE-LEHMAN, D., MURRAY, G.J., BOWERS, L.A., and BARRANGER, J.A. (1984). Isolation of cDNA clones for human β-glucocerebrosidase using the λGT11 expression system. Biochem. Biophys. Res. Commun. 123, 574–580.

GINNS, E.I., CHOUDARY, P.V., TSUJI, S., MARTIN, B.M., STUBBLEFIELD, B., SAWYER, J., HOZIER, J., and BARRANGER, J.A. (1985). Gene mapping and leader polypeptide sequence of human glucocerebrosidase: Implications for Gaucher disease. Proc. Natl. Acad. Sci. USA 82, 7101–7105.

GRABOWSKI, G.A., GATT, S., KRUSE, J., and DESNICK, R.J. (1984). Human lysosomal β-glucosidase: Kinetic characterization of the catalytic, aglycon and hydrophobic binding sites. Arch. Biochem. Biophys. 231, 144–157.

GRABOWSKI, G.A., GOLDBLATT, J., DINUR, T., KRUSE, J., SVENNERHOLM, L., GATT, S., and DESNICK, R.J. (1985a). Genetic heterogeneity in Gaucher disease: Physico-

kinetic and immunologic studies of the residual enzyme in cultured fibroblasts from non-neuronopathic and neuronopathic patients. Am. J. Med. Genet. 21, 529–549.

GRABOWSKI, G.A., DINUR, T., OSIECKI, K.M., KRUSE, J.R., LEGLER, G., and GATT, S. (1985b). Gaucher disease types 1, 2, and 3: Differential mutations of the acid β-glucosidase active site identified with conduritol B epoxide derivatives and sphingosine. Am. J. Hum. Genet. 37, 499–510.

GRAHAM, F.L., and VAN DER EB, A.J. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52, 456–467.

GRAVES, F.N., GRABOWSKI, G.A., LUDMAN, M.D., PALELE, P., and SMITH, F.I. (1986). Human acid beta-glucosidase: Northern blot and S1 nuclease analysis of mRNA from Hela cells and normal and Gaucher disease fibroblasts. Am. J. Hum. Genet. 39, 763–774.

HINK, W.F. (1970). Established insect cell line from the cabbage looper, Trichoplusia ni. Nature 226, 466–467.

JONSSON, L.M.V., MURRAY, G.J., GINNS, E.I., STRIJLAND, A., SCHRAM, A.W., TAGER, J.M., and BARRANGER, J.A. (1985). Processing of beta-glucocerebrosidase in normal human fibroblasts and fibroblasts from patients with Gaucher's disease. Fed. Proc. 44, 1742.

JONSSON, L.M.V., MURRAY, G.J., SORRELL, S., STRIJLAND, A., AERTS, J.F.G.M., GINNS, E.I., BARRANGER, J.A., TAGER, J.M., and SCHRAM, A.W. (1987). Biosynthesis and maturation of glucocerebrosidase in Gaucher fibroblasts. Eur. J. Biochem. 164, 171–179.

LEGLER, G. (1977). Glucosidases. Methods Enzymol. 46, 368–381.

MAEDA, S., KAWAI, T., OBINATA, M., FUJIWARA, H., HORIUCHI, T., SAEKI, Y., SATO, Y., and FURUSAWA, M. (1985). Production of human α-interferon in silkworm using a baculovirus vector. Nature 315, 592–594.

MATSUDAIRA, P. (1987). Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. 262, 10035–10038.

MATSUURA, Y., POSSEE, R.D., OVERTON, H.A., and BISHOP, D.H.L. (1987). Baculovirus expression vectors: The requirements for high level expression of proteins, including glycoproteins. J. Gen. Virol. 68, 1233–1250.

MURRAY, G.J., DOEBBER, T.W., WU, M.S., POMPIPOM, M.M., BUGIANESI, R.L., SHEN, T.V., and BARRANGER, J.A. (1985). Targeting of synthetically glycoslyated human placental glucocerebrosidase. Biochem. Med. Metab. Biol. 34, 241–246.

OKAYAMA, H., and BERG, P. (1983). A cDNA cloning vector that permits expression of cDNA inserts in mammalian cells. Mol. Cell Biol. 3, 280–289.

PATRICK, A.D. (1965). A deficiency of glucocerebrosidase in Gaucher's disease. Biochem. J. 97, 17c–18c.

PETERS, S.P., COYLE, P., and GLEW, R.H. (1976). Differentiation of beta-glucosidase in human tissues using sodium taurocholate. Arch. Biochem. Biophys. 175, 569–582.

REINER, O., WILDER, S., GIVOL, D., and HOROWITZ, M. (1987). Efficient in vitro and in vivo expression of human glucocerebrosidase cDNA. DNA 6, 101–108.

SHEARES, B.T., and ROBBINS, P.W. (1986). Glycosylation of ovalbumin in a heterologous cell: Analysis of oligosaccharide chains of the cloned glycoprotein in mouse L cells. Proc. Natl. Acad. Sci. USA 83, 1993–1997.

SMITH, G.E., JU, G., ERICSON, B.L., MOSCHERA, J., LAHM, H.W., CHIZZONITE, R., and SUMMERS, M.D. (1985). Modification and secretion of human interleukin 2 pro-

duced in insect cells by a baculovirus expression vector. Proc. Natl. Acad. Sci. USA 82, 8404–8408.

SORGE, J., WEST, C., WESTWOOD, B., and BEUTLER, E. (1985). Molecular cloning and nucleotide sequence of human glucocerebrosidase cDNA. Proc. Natl. Acad. Sci. USA 82, 7289–7293.

SUMMERS, M.D., and SMITH, G.E. (1987). A manual of methods for baculovirus vectors and insect cell culture procedures. Tex. Agric. Exp. Stn. Bull. No. 1555.

TAKASAKI, S., MURRAY, G.J., FURBISH, F.S., BRADY, R.O., BARRANGER, J.A., and KOBATA, A. (1984). Structure of the N-asparagine-linked oligosaccharide units of human placental β-glucocerebrosidase. J. Biol. Chem. 259, 10112–10117.

TARENTINO, A.L., GOMEZ, C.M., and PLUMMER, T.H., JR. (1985). Deglycosylation of asparagine-linked glycans by peptide: N-glycosidase F. Biochemistry 24, 4665–4671.

TRIMBLE, R.B., and MALEY, F. (1984). Optimizing hydrolysis of N-linked high mannose oligosaccharides by Endo-N-acetylglucosaminidase-H. Biochemistry 141, 515–522.

TSUJI, S., CHOUDARY, P.V., MARTIN, B.M., WINFIELD, S., BARRANGER, J.A., and GINNS, E.I. (1986). Nucleotide sequence of cDNA containing the complete coding sequence for human lysosomal glucocerebrosidase. J. Biol. Chem. 261, 50–53.

TSUJI, S., CHOUDARY, P.V., MARTIN, B.M., BARRANGER, J.A., STUBBLEFIELD, B.K., MAYOR, J.A., and GINNS, E.I. (1987). A mutation in the human glucocerebrosidase gene in neuronopathic Gaucher disease. N. Engl. J. Med. 361, 570–575.

TSUJI, S., MARTIN, B.M., BARRANGER, J.A., STUBBLEFIELD, B., LAMARCA, M.E., and GINNS, E.I. (in press). Genetic heterogeneity in type 1 Gaucher disease: Multiple genotypes in ashkenazic and non-ashkenazic individuals. Proc. Natl. Acad. Sci. USA.

WAHL, G.M., STERN, M., and STARK, G.R. (1979). Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. USA 76, 3683–3687.

WENGER, D.A., CLARK, C., SATTLER, M., and WHARTON, C. (1978). Synthetic substrate β-glucosidase activity in leukocytes: A reproducible method for the identification of patients and carriers of Gaucher's disease. Clin. Genet. 13, 145–153.

WILLEMSEN, R., VAN DONGEN, J.M., GINNS, E.I., SIPS, H.J., SCHRAM, A.W., TAGER, J.M., BARRANGER, J.A., and REUSER, A.J.J. (1987). Ultrastructural localization of glucocerebrosidase in cultured Gaucher's disease fibroblasts by immunocytochemistry. J. Neurol. 234, 44–51.

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